International Journal of Biological Macromolecules 119 (2018) 1164–1170

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: http://www.elsevier.com/locate/ijbiomac

A novel GH16 beta-agarase isolated from a marine bacterium, *Microbulbifer* sp. BN3 and its characterization and high-level expression in *Pichia pastoris*

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ARTICLE INFO

Article history:
Received 12 October 2017
Received in revised form 20 June 2018
Accepted 10 August 2018
Available online 11 August 2018

Keywords:
Beta-agarase
Microbulbifer sp.
Pichia pastoris
Expression

ABSTRACT

An agar-degrading bacterium, strain BN3, was isolated from a coastal soil sample collected in Taiwan Strait, China and identified to be a novel species of the genus *Microbulbifer*. The gene (N3-1) encoding for a novel β-agarase from the isolate was cloned and sequenced. It encoded a mature protein with 274 amino acids and a calculated molecular mass of 34.3 kDa. The deduced amino acid sequence manifested sequence similarity (61–84% identity) to characterized β-agarases in the glycoside hydrolase family 16. The recombinant agarase was hyper-produced extracellularly using *Pichia pastoris* as the host. After induction in a shake flask for 96 h, the yield of recombinant N3–1 protein reached 0.406 mg/mL, and the enzyme activity attained 502.1 U/mL. The enzyme purified by ion exchange chromatography displayed a specific activity of 6447 U/mg at pH 6.0 and 50 °C. The optimal pH and temperature for agarase activity were approximately 6 and 50 °C, respectively. The pattern of agarose hydrolysis showed that the enzyme was an endo-type β-agarase, capable of hydrolyzing agarose and *Gracilaria lemaneiformis*, with neoagarobiose and neoagarotetraose as the final main products.

1. Introduction

Agar, a polysaccharide present in the cell walls of red algae, consists of two different components, namely agarose and agaropectin [1]. Agarases, which can hydrolyze agar into oligosaccharides or monosaccharides, were found in agarolytic bacteria and have been isolated mostly from marine sediments, marine algae, and marine mollusks [2]. According to the cleavage pattern, agarases are classified into α-agarase (E.C. 3.2.1.158) and β-agarase (E.C.3.2.1.81). β-agarases recognize and hydrolyze the β-(1,4) glycosidic bonds between agarose repetition moieties (G-LA) in agar to produce neoagaro-oligosaccharides [3].

Beta-agarases have been reported to hydrolyze agar to yield NA2 (neoagarobiose) [4,5] A4 (neoagarotetraose) [6–8] and NA6 (neoagarohexaose) [9] as the main products. Neoagaro-oligosaccharides have many advantages, such as exhibiting a low-calorific value, an inhibitory action on bacterial growth and starch degradation [10], a moisturizing effect on the skin, and a whitening effect on melanoma cells [11,12]. Thus, neoagaro-oligosaccharides have a great impact on the health-food, pharmaceutical and cosmetic industries [11]. Searching for agarases with scientifically meaningful attributes is a prerequisite for preparing highly value-added neoagaro-oligosaccharides.

The catalytic domains of β-agarases are classified into several GH families, including GH-16, GH-50, GH-86, GH-96 and GH-118 [13–16], according to amino acid sequence similarities. GH-16 is the largest family. The structures [17,18] and biochemical properties of agarases in the GH16 family have been well studied [19–23]. These enzymes were expected to have distinctive action patterns or different specificities on natural agars because of the divergence of amino acid sequences. However, most of the GH16 β-agarases tend to hydrolyze agars to oligosaccharides with a larger degree of polymerization than neoagarobiose despite their heterogeneous enzymatic properties [17,18].

To investigate the diversity and application of agarases, we have isolated a number of agar-degrading microorganisms from the ocean and sequenced the genes encoding agarases and characterized the enzymes. In this report, we describe an agarase from a newly-identified agar-degrading bacterium, *Microbulbifer* sp. BN3, identified from marine algae collected in China, and elucidated the sequence of the gene encoding the agarase (N3-1). Moreover, we show that the recombinant agarase, which was expressed at a high level in *Pichia pastoris*, was

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https://doi.org/10.1016/j.ijbiomac.2018.08.053
0141-8130/

thermostable and capable of producing neoagarobiose and neoagarotetraose efficiently.

2. Materials and methods

2.1. Materials

pMD-18 T vector Kit and Genome Walking Kit were obtained from TAKARA (Dalian, China). The expression vector pPIC9K, *E. coli* Top10 and *P. pastoris* host strain GS115 were purchased from Invitrogen (USA).

DNA Gel Extraction Kit and other reagents were purchased from Sangon Biotech (Shanghai) Co., Ltd. (China). Culture media such as Luria Bertani (LB), Yeast extract-Peptone-Dextrose (YPD), Regeneration Dextrose medium (RD), and buffered methanol-complex medium were prepared as described in the *Pichia* Expression Kit User Manual. Primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd. (China) and sequencing reactions were performed by Invitrogen (Guangzhou, China). All other chemicals and reagents were of analytical grade.

2.2. Methods

2.2.1. Isolation and phylogenetic analysis of strain BN3

A coastal soil sample was collected from Taiwan Strait, China. The sample was diluted in artificial seawater, and spread on an artificial seawater agar plate supplemented with 1.5% (w/v) agar, yeast extract 1% (w/v) and Bacto peptone 0.3% (w/v). After incubation at 28 °C for 2 days and staining with Lugol's iodine solution (0.05 M iodine in 0.12 M KI), agarose-positive colonies with transparent zones were identified. The thermotolerant agarose-positive colonies were then selected by reheating the plating temperature to 40 °C for 2 or 3 days [24].

The 16S rRNA gene was amplified from the genomic DNA of positive strain by using bacterial universal primers, 27 F (5′-AGAGTTTGATCCTG GCTCAG-3′) and 1492R (5′-TACCTTGTTACGACTT-3′). Sequence alignments for the 16S rRNA gene were performed using ClustalW software, and phylogenetic analyses were performed by using the neighbor-joining (NJ) method in the Phylogeny Inference Package (PHYLIP) suite of programs [25,26].

2.2.2. Cloning of the β-agarase gene by genome walking

Alignment of the amino acid sequences derived from GH16 β-agarase genes of several microbial species (*Pseudomonas* sp., *Vibrio* sp., *Microbulbifer elongatus*, *Microbulbifer thermotolerans*, and *Saccharophagus degradans*, etc. obtained from the Genbank database) enabled the identification of highly conserved sequence regions. Based on the sequences of these regions, two degenerate primers P1 and P2 (Table 1) were synthesized. Touchdown PCR was initiated at 94 °C for 30 s. The annealing temperature of the reaction decreased from 60 °C to a touchdown 41 °C at the cooling rate of 1 °C in every cycle, followed by 20 cycles at 40 °C, 1.5 min at 72 °C. The gene sequence encoding conserved domain was subcloned into pMD-18 T, sequenced, and then a blast search in NCBI was conducted.

The flanking regions of the target gene fragments were cloned using a genome walking kit (TAKARA). All specific primers used in this study were designed based on the sequences of the core region obtained from touchdown PCR with degenerate primers (Table 1). The PCR products were purified on 1.0% (w/v) agarose gels, and the tertiary PCR products of the expected sizes were purified and cloned into pMD-18 T vector for sequencing.

Table 1
Primers used in this study.

Primers	Sequences
P1	5′-TGGARKCYTACGGCAGYGACCG-3′
P2	5′-GGYTTRTARAABCGHACCCAGTC-3′
N3-33	5′-GTGCCCTATGCGGAAATGGTCAAAG-3′
N3-32	5′-CCCCTGGTTTGTAGGCGTGCATATG-3′
N3-31	5′-GAGTTTAGCTTCGGCTCCGACGATC-3′
N3-53	5′-ACTTCACCTGCAATGCTTTGCCACC-3′
N3-52	5′-GGAGAATTCACTCCAGTCCCAGGCC-3′
N3-51	5′-ACCAGGCCGCGGGTATAGAAATCAC-3′

2.2.3. Heterologous expression of N3-1 in Pichia pastoris

Gene encoding a matured β-agarase (N3-1) was ligated with pPIC9K. The pPIC9K-N3-1 plasmid was constructed and transformed into GS115 cells by electroporation according to procedures in the *Pichia* Expression Kit User Manual. Yeast transformants were screened on RD (1 mol/L sorbitol, 2% (w/v) dextrose, 1.34% (w/v) YNB, 4×10^{-5}% (w/v) biotin, 0.005% (w/v) amino acids] plates after incubation at 28 °C for 2–4 days. Genomic DNA of the transformants was extracted for PCR verification of integration of N3-1 into the GS115 chromosome.

The positive-colonies were inoculated in YPD liquid medium and incubated overnight at 28 °C with shaking. Small-scale of fermentation for N3-1 expression induction was performed in 100 mL Erlenmeyer flasks following the instructions in the *Pichia* Expression Kit User Manual. Samples (0.5 mL) were taken every 24 h, meanwhile methanol was added to 1% (v/v) daily during fermentation. The samples were centrifuged, and the supernatants were used for enzyme assay and SDS-PAGE analysis [27,28].

2.2.4. Purification of recombinant N3-1

The recombinant N3-1 in culture medium was first concentrated by addition of 55% (*w*/*v*) saturated ammonium sulfate [$(NH_4)_2SO_4$] followed by centrifugation at 13,200 ×*g* and 4 °C for 30 min. The resulting precipitate was resuspended in 50 mmol/L Tris-HCl buffer (pH 8.0), desalted using a HiTrap™ desalting column (GE Health, USA), and then purified with a 5 mL HiTrap™ DEAE FF column. The purity of the enzyme was established by SDS-PAGE analysis.

2.2.5. Enzyme analysis and protein quantification

Protein concentration was determined with a Bradford Protein Quantification Kit (Sangon, China). β-agarase activity was measured using 0.3% agar (Sigma G0753) as the substrate. One unit of β-agarase activity was defined as the amount of enzyme required to release 1 μmol of reducing sugar at 50 °C and pH 6.0. [29,30]

2.2.6. Effects of pH and temperature on the recombinant N3-1

The optimum pH for N3-1 activity was determined by measuring the activity over the pH range of 4.0–9.0 at 50 °C. The buffers used were 50 mmol/L NaH_2PO_4-citric acid, pH 4.0–8.0; and 50 mmol/L glycine-NaOH pH 8.0–9.0. Enzyme stability at various pH values was studied by measuring the residual enzyme activity at 50 °C after incubation of agarase at room temperature for 1 h in buffers with pH ranging from 4.0 to 9.0.

The optimum temperature for N3-1 activity was ascertained by performing the assay in 50 mmol/L NaH_2PO_4-citric acid buffer (pH 6.0) at various temperatures in the range of 30–70 °C. The thermal stability of agarase was estimated by analyzing the residual enzyme activity at 50 °C and pH 6.0 after incubating enzyme preparations in 50 mmol/L NaH_2PO_4-citric acid buffer (pH 6.0) at various temperatures (30–70 °C) for 30 min.

2.2.7. Effects of metal-ions and inhibitors on the activity of recombinant N3-1

The activity of the purified recombinant N3-1 was determined after incubation in 50 mmol/L NaH_2PO_4-citric acid buffer (pH 6.0) in the presence of various metal-ions and inhibitors at 2 mmol/L concentration at 37 °C for 30 min. The residual activity was measured in 50 mmol/L NaH_2PO_4-citric acid buffer (pH 6.0) at 50 °C. The activities were compared with the control in the absence of metal ions and inhibitors.